Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes

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Summary

A model rumen system, dual-flow continuous culture fermenters, was evaluated by two comparative criteria in two experiments using ribosomal (r)RNA-targeted DNA probes to compare key microbial groups in samples. The initial experiment measured temporal changes in population structure during adaptation of ruminal microbial populations in fermenters over 240 h. The fermenter inoculum contained 34.9% Bacteria, 60.1% Eukarya and 6.8% Archaea measured as a fraction of total small subunit (SSU) rRNA quantified using a universal probe. The cellulolytic bacterial genus *Fibrobacter* comprised 9.5% of total SSU rRNA in the inoculum. After 240 h of fermenter operation, the average abundance was 80.9% Bacteria, 6.1% Eukarya, 5.1% Archaea and *Fibrobacter* genus accounted for 6.6% of the total SSU rRNA. Divergence between ruminal and fermenter population structure was evaluated in the second experiment and samples were classified as ruminal, inoculum or fermenter (96, 120, 144 and 168 h of fermenter operation). Fermenter samples had higher relative abundances of Bacteria (84.5%) and Archaea (2.1%) and lower relative abundances of Eukarya (1.8%) than ruminal samples (average 48.0% Bacteria, 1.3% Archaea and 61.5% Eukarya). The relative abundance of *Fibrobacter* was similar in all samples, averaging 2.5%. The ruminal and fermenter samples had similar proportions of *F. succinogenes* and *F. succinogenes* subgroup 3 (as a percentage of *Fibrobacter* SSU rRNA). *Fibrobacter succinogenes* subgroup 1 and *F. intestinalis* proportions of *Fibrobacter* were lower in fermenter samples (8.2% and 0.7% respectively) than in ruminal samples (28.4% and 2.2% respectively). Fermenters were able to maintain a core prokaryotic community structure similar to the native microbial community in the rumen. Although protozoa populations were lost, maintenance of *Fibrobacter* and archaeal populations indicated that the model system supported a functional community structure similar to the rumen. This model rumen system may serve as a suitable tool for studying aspects of ruminal microbial ecology and may resolve some of the relationships between microbial community structure and function by providing control of experimental conditions.

Introduction

A natural history of microoganisms, an essential prelude to more refined ecological studies, does not exist in a form recognizable to macroecologists. The limited ability of the microbial ecologist to directly observe specific organisms in their environment is a major constraint to the study of microbial ecology (Atlas, 1986). As a result, the study of microbial ecology is one of the least developed and quantified areas of research in microbiology (Karl, 1986). Pure culture studies may bias understanding of microbial ecology because characteristics and activities measured in microbial monoculture are not necessarily representative of *in situ* responses (Karl, 1986). Furthermore, organisms isolated and raised in culture do not necessarily represent predominant populations.

Although the rumen is among the better-characterized microbial habitats in terms of identification of species present, substrate utilization and metabolism (Hungate, 1966; Russell, 1983), little is known about the ruminal microbial population and trophic structure (Stewart and Bryant, 1988; Dehority, 1993). The study of ruminal

microbial ecology is complicated by daily fluctuations in the environment, microenvironments within the rumen and the effects of animal physiology on the rumen. Further complications arise from the considerable amount of overlap in substrate degradation capability and a large degree of cross-feeding among ruminal microoganisms (Warner, 1956; Russell, 1983; Stewart and Bryant, 1988).

The complexity of ruminal microbial ecology has resulted in variable and sometimes conflicting results from efforts to manipulate ruminal fermentation (Van Nevel and Demeyer, 1988). Changes in diet have resulted in alterations of microbial populations, determined by using selective culture media (Russell, 1983; Stewart and Bryant, 1988; MacGregor *et al.*, 2000), but these results do not necessarily give a clear picture of whether or not the overall microbial community structure is affected (Attwood *et al.*, 1988; Stahl *et al.*, 1988; Stewart and Bryant, 1988). Although the animal influence is thought to be less significant than microbial contributions, the relationship between microbial and animal contribution to fermentation and microbial community structure is not well understood, further complicating experimentation. Efforts to alter ruminal fermentation will not be fully successful until the complexity of the ruminal microbial community is understood more completely (Van Nevel and Demeyer, 1988).

An *in vitro* system that satisfactorily models the rumen would benefit the study of dietary effects on microbial community structure and offer control of the physical and chemical environment needed for detailed experimentation. To be an adequate experimental tool, a model rumen should mimic the natural rumen in a number of ways including the physical environment (temperature, pH, gas phase and turnover rates) and the maintenance of key microbial populations (including diversity and concentrations) (Warner, 1956). Other important criteria that should be met in these models include sustained digestion of substrates and comparable fermentation product evolution. The dual-flow continuous culture fermenter system represents a model rumen that may meet these criteria. However, it is important to document more fully the comparability between the natural and model system. Research examining ruminal microbial community structure and the multitude of interactions within that community has been, for the most part, unfeasible because of the labour, time and variability associated with cultural techniques. Thus, little is known about ruminal microbial community structure and complex interactions (Stewart and Bryant, 1988; Dehority, 1993).

The application of molecular techniques to microbial ecology studies offers several advantages over conventional culture-based techniques. Oligonucleotide probe methods are highly specific, allow for investigation of several microbial populations simultaneously and give higher resolution than traditional metabolic tests (Attwood *et al.*, 1988; Stahl *et al.*, 1988). Nucleic acids can be extracted directly from the *in situ* biomass without the need for prior culturing of species (Pace *et al.*, 1986; Attwood *et al.*, 1988; Stahl *et al.*, 1988). This is advantageous because it allows a direct comparison of microbial populations and quantification of population changes as environmental conditions are altered. In the rumen, the problem of isolating species that attach to plant cell walls is also eliminated because nucleic acids can be extracted from organisms that are attached to the substrate (Odenyo *et al.*, 1994).

To facilitate the study of the microbial ecology of the rumen, an *in vitro* system was evaluated as a model of ruminal microbial ecology. The current studies addressed the critical need for further validation of the model by characterizing population structure, using molecular measures, in relationship to more conventional measures [e.g. volatile fatty acids (VFAs), digestion]. Use of molecular probes offers a method for studying complex microbial communities in their environments at a higher resolution than that offered by cultural techniques (Stahl *et al.*, 1988; Raskin *et al.*, 1997). We targeted key microbial populations for quantification using DNA probes in order to obtain more detail of the ruminal microbial community as an essential part of the validation of the model system. The results presented here follow the adaptation of microbial populations to the model rumen (through 240 h of operation) and a comparison of microbial populations in the rumen and fermenters.

Results

Adaptation of microbial populations to model rumen

This first experiment followed the adaptation of key microbial groups from the rumen over 240 h of fermenter operation. Nutrient digestibilities (organic matter digestion 54.2%, acid detergent fibre 28.2% and crude protein 60.3%, average values) were used as a check of fermenter operation and were within normal ranges. The pH of the ruminal sample was 6.32. The ruminal sample had an ammonia concentration of 23.1 mg 100 ml^{-1}, while the average in fermenters during the last 3 days of operation was 16.6 mg 100 ml^{-1}. The total concentration of VFAs averaged 99.0 mM for all samples. Proportions of VFAs were similar between rumen and 240 h fermenter samples; during the course of fermenter operation, molar proportions averaged 68.3, 17.3 and 10.3 mol per 100 mol for acetate, propionate and butyrate, respectively, in the rumen and 66.0, 21.9 and 9.0 mol per 100 mol in the fermenters.

The amount of SSU rRNA extracted (μg of rRNA g^{-1} sample) and the relative abundance of microbial populations for ruminal and fermenters (0 and 240 h) are

Table 1. Relative abundance of microbial populations and amount of rRNA extracted from ruminal and dual-flow continuous culture fermenter samples.

Population	Rumen	Fermenter[a] 0 h[b]	240 h	SD[c]	Rumen	Fermenter 0 h	240 h	SD
	μg SSU rRNA g^{-1} sample				% of total SSU rRNA			
Total	212.8	163.5	113.9	51.1				
Bacteria[d, e]	74.3	55.9	93.2	62.4	34.9	33.9	80.9	9.2
Eukarya[f, g]	127.9	63.8	6.3	12.8	60.1	39.0	6.1	3.5
Archaea[f]	14.4	4.9	5.3	2.9	6.8	3.0	5.1	1.2
Fibrobacter	20.3	7.3	6.8	5.1	9.5	4.5	6.6	3.3

a. Value is the mean of four fermenters.
b. Time of fermenter operation (h).
c. Standard deviation.
d. Amount and relative abundance of Bacteria include *Fibrobacter*.
e. Relative abundance greater in 240 h fermenter samples than in ruminal, $P \leq 0.05$.
f. Amount of SSU rRNA extracted lower in 0 and 240 h fermenter samples than in ruminal, $P \leq 0.05$.
g. Relative abundance lower in 0 and 240 h fermenter samples than in ruminal, $P \leq 0.05$.

presented in Table 1. The concentration of eukaryotic and archaeal rRNA was significantly lower in the fermenters at 0 and 240 h than in the ruminal sample. The relative abundance of Eukarya was also lower in the fermenters; however, archaeal abundance in fermenter samples was not significantly different from the ruminal sample.

Analysis of variance revealed no statistically significant differences among fermenters for microbial populations. Therefore, data presented in the following discussion are the average of the four fermenters. Bacterial and eukaryotic populations significantly changed over time, but archaeal and *Fibrobacter* populations did not, both for relative abundance and amount of rRNA. Microbial group relative abundances in the ruminal sample were 34.9% bacterial, 60.1% eukaryotic, 6.8% archaeal and 9.5% *Fibrobacter* SSU rRNA (percentage of total SSU rRNA). By 6 h, bacterial SSU rRNA relative abundance increased

(Fig. 1), while the eukaryotic SSU rRNA abundance decreased (Fig. 1) compared with the rumen. After 240 h of fermenter operation, bacterial SSU rRNA represented 80.9% of total SSU rRNA and eukaryotic SSU rRNA was 6.1%. The decrease in eukaryotic abundance was supported by microscopic counts of protozoa. Counts were 56, 20, 10, 2 and 0.5 (1×10^4 protozoa ml^{-1}) at 0, 6, 12, 24 and 48 h respectively. By 72 h, and over 240 h, no protozoa were observed in any fermenter and the average proportion of eukaryotes was 7.4%. Abundance of *Fibrobacter* (Fig. 1) averaged 6.9% of the total SSU rRNA throughout fermenter operation.

Comparison of model and natural rumens

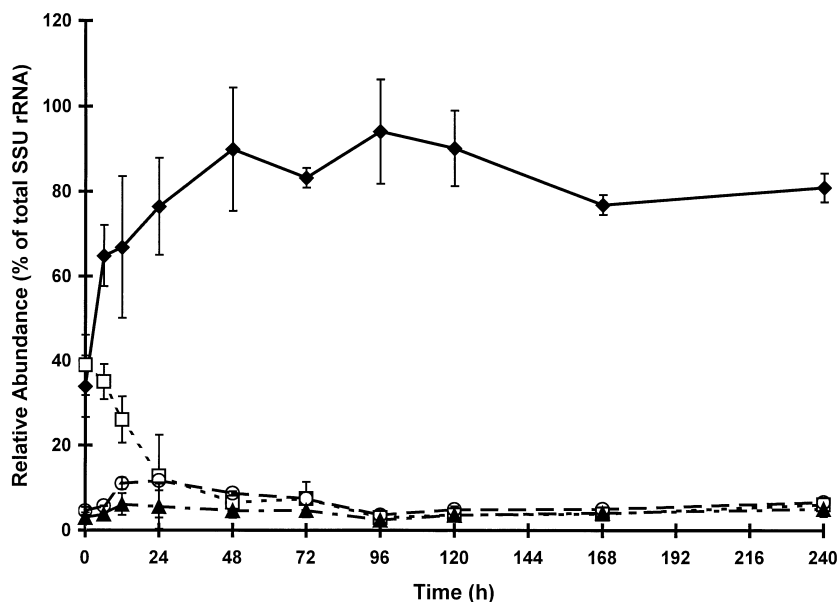The second experiment was a more detailed comparison of microbial populations in the model and natural rumen

Fig. 1. Abundance of bacterial (—), eukaryotic (–), archaeal (— – —) and *Fibrobacter* (— —) SSU rRNA (% of total SSU rRNA) in dual-flow continuous culture fermenters ($n = 4$) from 0 to 240 h of operation. Error bars are the standard error of the mean for each time-point.

with fermenter sampling times based on the results of the first experiment. The variation among the ruminal samples taken on four consecutive days in parallel to fermenter operation was low in both fermentation characteristics and microbial population measures. The data presented for the rumen are averages of the four samples taken on consecutive days. Chemical fermentation characteristics were similar between ruminal and fermenter samples, although total VFA concentration tended to be higher in fermenter samples than ruminal samples ($P = 0.07$) (Table 2). Lactate was not detected in any sample. Ruminal pH was higher than the pH maintained in the fermenters (Table 2). Proportions of acetate and branched chain fatty acids (sum of isobutyrate, isovalerate and 2-methylbutyrate) were higher and valerate was lower in the ruminal than in the fermenter samples (Table 2).

Based on statistical analyses, there were no differences among fermenters for chemical fermentation characteristics or microbial populations. For the following results and discussion, fermenter samples are represented by sampling time (96, 120, 144 and 168 h) and are means of the four fermenters. The amount of SSU rRNA extracted was not significantly different among ruminal, inoculum and fermenter samples (Table 3). The relative abundances (% of total SSU rRNA) of Bacteria, Eukarya, Archaea, Gram-positive bacteria and *Fibrobacter* were similar in ruminal and inoculum samples (Table 4, Figs 2 and 3). Fermenter samples (96, 120, 144 and 168 h) had a higher relative abundance of bacteria (averaging 84.5%) and a lower relative abundance of Eukarya (averaging 1.8%) than either ruminal or inoculum samples (Table 3, Fig. 2). Archaeal relative abundance was higher in 96, 120, 144 and 168 h samples (2.0% of total SSU rRNA, average for all fermenter samples) than in ruminal samples (1.3% of total SSU rRNA) (Table 3, Fig. 3).

Table 2. Chemical fermentation characteristics in ruminal and fermenter samples.

Measure	Rumen	Fermenter	SEM[a]	P =
pH	6.56	6.25		NA[b]
Ammonia nitrogen (mg 100 ml^{-1})	14.6	17.9	2.5	NS[c]
Total VFA (mM)	98.1	122.8	7.9	0.07
Individual VFA (mol 100 M^{-1})				
Acetate	68.7	63.3	1.0	0.02
Propionate	15.4	19.0	1.7	NS
Butyrate	11.3	13.6	1.1	NS
Valerate	1.9	2.7	0.1	0.004
Branched chain[d]	2.6	1.4	0.3	0.03

a. SEM, standard error of the mean.
b. NA, not analysed.
c. NS, not significant ($P > 0.05$).
d. Branched chain, sum of isobutyrate, isovalerate and 2-methyl butyrate.

Abundance of Gram-positive bacteria was higher in the fermenter samples (37.2% average for all fermenter samples) than in the inoculum samples (24.4% of total SSU rRNA) (Table 3, Fig. 2). The relative abundance of *Fibrobacter* in the fermenter samples (2.5%) was similar to the proportion found in ruminal and inoculum samples (Table 3, Fig. 3).

The abundance of *Fibrobacter succinogenes* (% of *Fibrobacter* SSU rRNA) in ruminal and fermenter samples did not differ; however, the proportion of *F. succinogenes* in inoculum samples tended to be lower than rumen 120 and 144 h samples (Table 3, Fig. 4). Proportions of *F. intestinalis* and *F. succinogenes* subgroup 1 (% of *Fibrobacter* SSU rRNA) did not differ between rumen and inoculum samples, but were decreased in fermenter samples (Table 3, Fig. 4). There was a great deal of variability in the proportion of *F. succinogenes* subgroup 3 (% of *Fibrobacter* SSU rRNA) among all samples (especially fermenter samples) and no statistical differences were detected (Table 3, Fig. 4).

Discussion

Probe data evaluation

An important aspect of using molecular probes is to have some way of evaluating how completely microbial populations are circumscribed by the probes. The use of phylogenetically based hybridization probes serves this need. For example, if the domain probes circumscribe all organisms present, then the sum of the domain probes should equal the total, as determined with the universal probe. In this regard, our general probe quantification data were consistent with the domain probes summation, less than 10% of observations in both experiments were outside $100 \pm 20\%$. Given the many caveats associated with the use of DNA probes, the use of domain probes can serve as a procedural check and as the framework for further description of the microbial community structure. An additional caution for interpreting molecular probe data must be interjected. Microbial population data are presented in other research, for the most part, as relative abundance of total rather than as actual individual quantities of SSU rRNA and may not always correspond to change in absolute abundance. For example, if the amount of total SSU rRNA increases and the amount of eukaryotic SSU rRNA remains constant, the relative abundance of Bacteria will increase and the relative abundance of Eukarya will decrease. However, this does not represent a 'true' decrease in Eukarya. Presentation or discussion of the amount of SSU rRNA extracted from, as well as the relative abundance of, microbial populations, as included in this study, may help prevent misinterpretations.

Table 3. Relative abundance of microbial populations and amount of rRNA extracted from ruminal, inoculum and fermenter (96, 120, 144 and 168 h) samples.

Population	Rumen	Inoculum	Fermenter[a] 96 h	120 h	144 h	168 h	SEM[b]
μg g^{-1} sample							
Total SSU rRNA[c]	124.1	79.4	174.9	154.8	274.1	180.7	46.5
% of total SSU rRNA							
Bacteria[c,d,e]	48.0	52.3	84.0	84.2	86.7	83.0	4.4
Eukarya[f,g]	52.2	70.7	1.7	1.1	2.0	2.5	8.6
Archaea[d]	1.3	1.8	2.3	2.1	1.8	2.1	0.2
Gram-positive bacteria[d]	28.0	24.4	39.6	33.8	37.5	38.0	4.0
Fibrobacter	2.2	2.8	2.2	3.4	2.2	2.5	0.3
% of *Fibrobacter* SSU rRNA							
Fibrobacter succinogenes	90.6	67.4	83.7	89.0	92.5	75.8	5.7
Fibrobacter intestinalis[f]	2.1	2.7	0.4	0.9	0.6	0.3	0.5
F. succinogenes subgroup 1[f,g]	28.4	29.7	7.9	9.4	9.0	6.5	3.6
F. succinogenes subgroup 3	25.3	46.5	13.5	39.6	21.5	9.3	9.2

a. Average of samples from four fermenters after 96, 120, 144, and 168 h of operation.
b. SEM = standard error of the mean.
c. Relative abundance of Bacteria includes *Fibrobacter* and Gram-positive bacteria.
d. Values for fermenter samples significantly greater than inoculum samples. Based on paired *t*-test, $P \geq 0.05$.
e. Values for fermenter samples significantly greater than ruminal samples. Based on paired *t*-test, $P \geq 0.05$.
f. Values for fermenter samples significantly less than ruminal samples. Based on paired *t*-test, $P \geq 0.05$.
g. Values for fermenter samples significantly less than inoculum samples. Based on paired *t*-test, $P \geq 0.05$.

Amount of SSU rRNA

The total amount of SSU rRNA extracted from all samples was relatively consistent for both studies, averaging 163.4 μg g^{-1} of sample in the adaptation experiment and 164.7 μg g^{-1} of sample in the comparison experiment. In both experiments, inoculum samples had lower amounts of SSU rRNA extracted (0 h, Table 1 and Inoculum, Table 3). This was supported by decreased amounts of bacterial, eukaryotic, archaeal, Gram-positive bacterial and *Fibrobacter* SSU rRNA in the inoculum samples. The inoculum is primarily the liquid fraction of ruminal contents, while both ruminal samples and fermenter samples include particulate matter. The majority of ruminal microorganisms are found in association with particulate matter in the rumen. About 70–80% of Bacteria are associated with feed particles, while 20–30% are found in the fluid (Cheng *et al.*, 1995). The smaller amount of particulate matter in the inoculum probably contributes to the lower amount of microbial SSU rRNA in
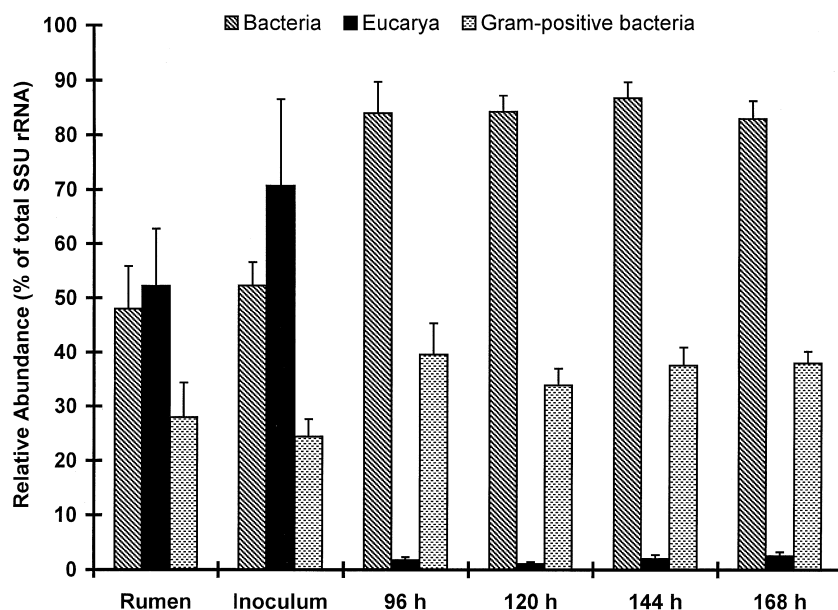

Fig. 2. Relative abundance (% of total SSU rRNA) of Bacteria, Eukarya and Gram-positive bacteria from ruminal, inoculum and fermenter (after 96, 120, 144 and 168 h of operation) samples. Each column is the mean of four samples and the error bar represents the standard error of the mean.

Fig. 3. Relative abundance (% of total SSU rRNA) of Archaea and *Fibrobacter* from ruminal, inoculum and fermenter (after 96, 120, 144 and 168 h of operation) samples. Each column is the mean of four samples and the error bar represents the standard error of the mean.
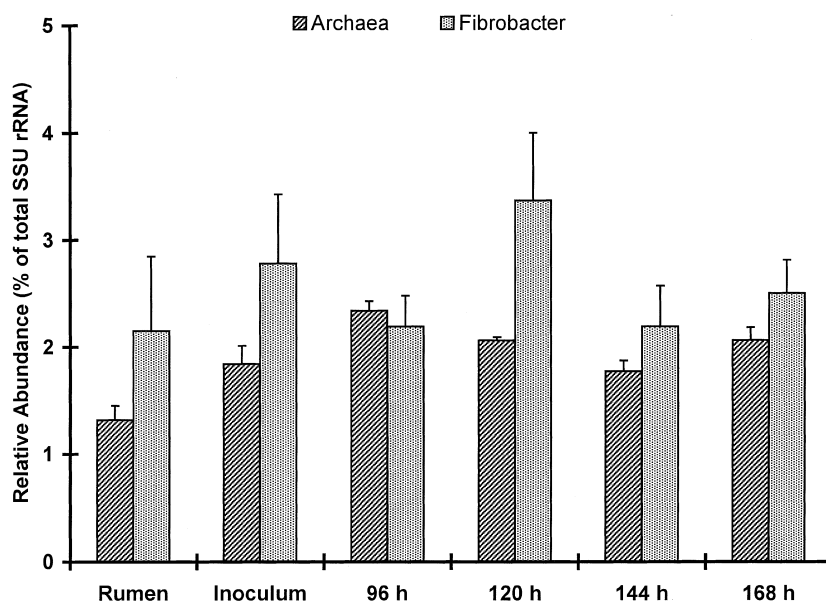
inoculum samples and explains the elevated proportion of Eukarya (Table 3). The similarity between ruminal and inoculum samples for relative abundances of Bacteria, Eukarya and Gram-positive bacteria suggests that, although there was less microbial biomass in the inoculum samples, gross community structure was not significantly altered.

System stability

Tracking of the adaptation of microbial populations to the model rumen over time allowed for determination of system stability. The most striking population change that

occurred during the initial fermenter runs was the decreased Eukarya. This occurred concurrently with an increase in Bacteria (both relative abundance and amount of SSU rRNA extracted from samples), which is an expected result of protozoa loss. Visual observation supported the loss of protozoa; decreases in protozoa have previously been observed in our system (Mansfield *et al.*, 1995) and in other rumen models (Slyter *et al.*, 1964; Prevot *et al.*, 1995). The loss of microscopically observable protozoa from our system was slightly faster than could be accounted for by the dilution rate of 0.1 h^{-1}. This may reflect an additional contribution to loss by lysis. In other work, dual-flow continuous culture fermenters
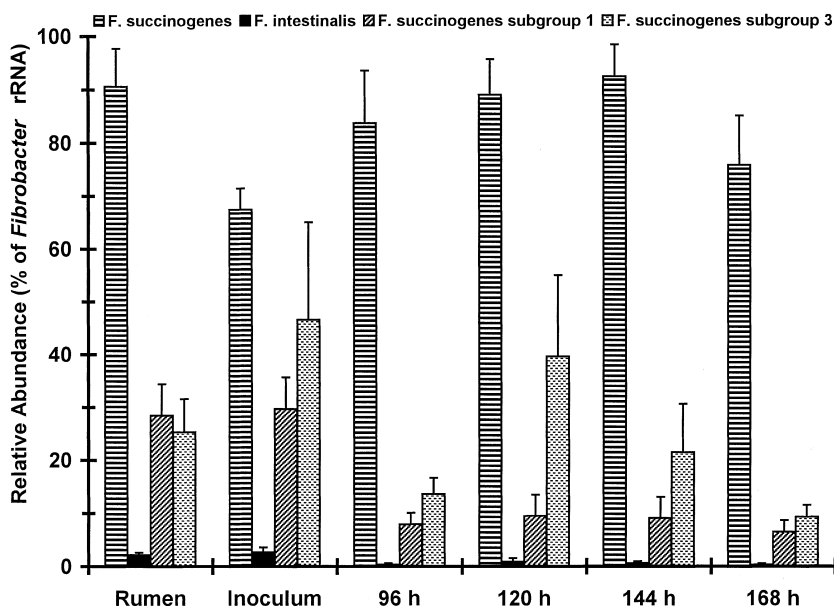

Fig. 4. Abundance of *Fibrobacter* populations (% of *Fibrobacter* rRNA): *F. succinogenes*, *F. intestinalis, F. succinogenes* subgroup1 and *F. succinogenes* subgroup 3 from ruminal, inoculum and fermenter (after 96, 120, 144 and 168 h of operation) samples. Each column is the mean of four samples and the error bar represents the standard error of the mean.

supported anaerobic fungi in numbers similar to those found *in vivo* (Mansfield *et al.*, 1995). Fungal population density in the rumen has been estimated between 10^3 and 10^5 ml^{-1} and the contribution to biomass estimated to be up to 8% of the total (Orpin and Joblin, 1988). Thus, it is reasonable to attribute the eukaryotic average of 6.1% at 240 h of the adaptation study and 2.5% at 168 h in the comparison study to anaerobic fungi. Although some systems have been able to maintain some protozoa, this was only achieved by decreasing liquid outflow, 0.06 and 0.03 h^{-1} in single-flow models compared with 0.1 h^{-1} for our dual-flow system, apparently allowing some of the protozoa to be retained (Slyter *et al.*, 1964). Extensive microbial community analysis has only been reported for the RUSITEC rumen model (Prevot *et al.*, 1995). While some ciliated protozoa were maintained in this system, it was at a lower concentration than observed *in vivo* (1×10^5 cells ml^{-1} *in vivo* to 1×10^3 cells ml^{-1} *in vitro*). In addition, this was achieved with a lower liquid ouflow rate (0.03 h^{-1}) and dry matter intake (8.8 g DM d^{-1}) than in our system (0.1 h^{-1} and 75 g DM d^{-1}). In the RUSITEC rumen model, while the predominant ruminal Bacteria were isolated and identified in the model, total Bacteria decreased almost 70% from 5×10^{10} cells ml^{-1} *in vivo* to 5×10^9 cells ml^{-1} *in vitro* (Prevot *et al.*, 1995).

Physical environment

The dual-flow continuous culture fermenter system has previously been demonstrated to meet the physical environment, substrate digestion and fermentation end-product criteria (Hoover *et al.*, 1976; Hannah *et al.*, 1986; Mansfield *et al.*, 1995). In the current study, some differences in chemical fermentation characteristics between ruminal and fermenter samples were observed. The tendency of total VFA concentration to be higher in the fermenter samples than in ruminal samples was not unexpected and has been previously observed in the dual-flow continuous culture fermenters (Hannah *et al.*, 1986; Mansfield *et al.*, 1995). The fact that VFAs are not absorbed from the glass fermenter flasks as they are across the rumen wall probably accounts for the higher VFA concentrations *in vitro*. Some of the differences in the proportion of individual VFAs may be related to the difference in pH between the rumen and fermenters. Ruminal pH was higher than the controlled pH in the fermenters. Decreased acetate and branched-chain VFAs and increased valerate have been observed when pH is decreased from 6.5 to 6.0 *in vitro* (Erfle *et al.*, 1982).

Microbial populations

Data comparing the microbial ecology of the rumen and fermenter system is not extensive. Mansfield *et al.* (1995) compared microbial populations in the fermenters and rumen using cultural techniques. Fermenters maintained amylolytic and proteolytic bacteria and anaerobic fungi in numbers similar to those found in the rumen. However, cellulolytic bacteria and protozoal numbers decreased, while total viable bacterial numbers increased in the fermenters compared with ruminal numbers (Mansfield *et al.*, 1995). The use of molecular techniques, in particular DNA probes targeted to SSU rRNA, allows for higher resolution investigations of microbial population dynamics (Stahl *et al.*, 1988; Lin *et al.*, 1994; Sharp *et al.*, 1998) and, thus, were well-suited as a means of comparing natural and model rumens in this study. The key functional roles of *Fibrobacter* and methanogens in the microbial food chain makes them important populations to measure in assessing ruminal microbial ecology. *Fibrobacter* is one of the primary cellulose-degrading bacteria in the rumen, participating in the initial steps of degradation of plant cell-wall material (Stewart and Bryant, 1988). Methanogens, on the other hand, by removal of the hydrogen generated by fermentation, participate in the final steps of fermentation promoting more complete oxidation of substrates and greater energy recovery by fermenting organisms (Stewart and Bryant, 1988; Raskin *et al.*, 1997). The expected changes in bacterial and archaeal abundance as a result of the loss of Eukarya would be 78.4% and 15.2% respectively (mathematical calculation based on change of bacterial + archaeal abundance $\approx$40% in rumen to 94% in fermenters at 24 h). These values differ from the observed values of 80.9% bacterial relative abundance and 5.1% archaeal abundance, supporting maintenance of functional community structure rather than simply reflecting a mathematical change in abundance because of loss of eukaryotic SSU rRNA.

Archaea. Relationships important to the maintenance of methanogenic populations appear to have been maintained; archaeal relative abundance and amount of SSU rRNA extracted were similar in the adaptation experiment and slightly higher in fermenter samples than in ruminal samples in the comparison experiment. All archaeal biomass in the rumen is thought to represent methanogens. While interspecies hydrogen transfer between protozoa and methanogens is a key component of CH_4 production in the rumen, H_2 is also produced by a great variety of ruminal anaerobic fungi and bacteria, such as *Ruminococcus* and *Butyrivibrio* (Stewart *et al.*, 1990). Because overall digestibility and fermentation patterns were not significantly altered by the loss of protozoa, it appears that other microbial groups replace them and, in so doing, retain similar relationships to methanogenic populations. This is consistent with the observation that defaunation does not appear to significantly alter ruminal

function (Dehority and Orpin, 1988). While overall archaeal abundance was maintained in fermenters, the predominant methanogen groups probably changed. Sharp *et al.* (1998) demonstrated that *Methanobacteriaceae* comprised 90% archaeal abundance associated with protozoa in ruminal samples. Although predominantly protozoa-associated, some *Methanobacteriaceae* were free-living; however, *Methanomicrobiales* were demonstrated to be exclusively free-living and comprised the greatest proportion of archaeal abundance in these fermenters (Sharp *et al.*, 1998).

Fibrobacter. Probes targeting *F. intestinalis*, *F. succinogenes*, *F. succinogenes* subgroup 1 and *F. succinogenes* subgroup 3 were selected to obtain a more detailed and higher resolution comparison of *Fibrobacter* populations in ruminal and fermenter samples. The amount of *Fibrobacter succinogenes* and *F. succinogenes* subgroup 3 SSU rRNA extracted and the proportion of *Fibrobacter* SSU rRNA did not differ between ruminal and fermenter samples. Inoculum samples had the lowest *F. succinogenes* relative abundance and higher abundance of *F. succinogenes* subgroup 3. It is unclear why the proportion of *Fibrobacter* accounted for by *F. succinogenes* in the inoculum samples was so much lower than in either ruminal or fermenter samples, but may be related to preferential attachment to particles. The lower amount of *F. intestinalis* SSU rRNA extracted and its lower proportion of *Fibrobacter* abundance is consistent with the hypothesized role of *F. intestinalis* as a lower gastrointestinal bacterium (Amann *et al.*, 1992), rather than a predominant ruminal bacterium. The proportion of *Fibrobacter* accounted for by *F. succinogenes* and *F. intestinalis* was never greater than 93.1% (144 h) and, excluding inoculum samples, averaged 87.2%. Unaccounted-for diversity within *Fibrobacter* has previously been documented for the horse caecum (Lin and Stahl, 1995) and has been suggested in studies of ruminal samples from goats (Lin *et al.*, 1994).

Fibrobacter subgroups. While the fermenters maintained *Fibrobacter* and *F. succinogenes* in similar proportions to the rumen, the subspecies proportions were different. In ruminal samples, *F. succinogenes* subgroups 1 and 3 accounted for almost equal proportions of *Fibrobacter*, 28.4 and 25.3% respectively. However, in inoculum and fermenter samples, *F. succinogenes* subgroup 3 accounted for a greater proportion of the *Fibrobacter* populations than *F. succinogenes* subgroup 1. The relative abundance of *F. succinogenes* subgroup 1 in all samples was similar to previous reports (Lin *et al.*, 1994). In the bovine rumen, Lin *et al.* (1994) reported that *F. succinogenes* subgroup 1 was consistently less abundant than *F. succinogenes* subgroup 2. Interestingly, *F. succinogenes*

S85, a commonly used strain in cultural studies, is included in subgroup 1 and strains in this subgroup do not appear to predominate in the bovine rumen. It is unclear why there was such high variability of *F. succinogenes* subgroup 3 in samples. Based on the results reported here and by Lin *et al.* (1994), it appears that *F. succinogenes* strains targeted by the subgroup 2 probe are the most abundant strains in the bovine rumen. However, owing to lack of data for subgroup 2 in the present study and for subgroup 3 in the previous study, in which no hybridization was observed (Lin *et al.*, 1994), this trend can only be inferred. It is possible that differences in environmental conditions, such as feed particle size or pH, between the rumen and fermenters were enough to change the proportions of the *F. succinogenes* subgroups maintained in the fermenters.

Gram-positive bacteria. Fermenter samples had the highest relative abundance of Gram-positive bacterial SSU rRNA and this followed the relative abundance of bacterial SSU rRNA. However, if Gram-positive bacterial abundance was calculated as a percentage of bacterial SSU rRNA, inoculum and fermenter samples had a lower proportion of Gram-positive bacterial SSU rRNA than ruminal samples (58.2 versus 46.6 and 44.1%, SEM = 6.9, for cow, inoculum and fermenter samples respectively). In previous reports, the proportion of bacteria classified as Gram positive, based on the Gram stain procedure, has generally been lower than 40%, except on very high grain diets. Nagaraja *et al.* (1978) reported 10.7% of bacteria stained Gram positive in ruminal fluid with a pH of 6.45. Gram-positive bacteria only accounted for 7% of culturable strains in a steer fed 90% forage and 16% in single-flow continuous culture fermenters (Slyter and Putnam, 1967). The difference between our results and the seemingly contradictory reports in the literature can be explained phylogenetically. *Selenomonas*, *Butyrivibrio* and *Megasphaera* genera stain Gram variable, but, based on comparative 16S rRNA sequencing, are affiliated with Gram-positive genera (Stackebrandt *et al.*, 1985). The Gram-positive probe that was used is inclusive of *Selenomonas*, *Butyrivibrio* and *Megasphaera* (MacGregor *et al.*, 2000). *Selenomonas* and *Butyrivibrio* species are generally among the most numerous bacteria in the rumen (Slyter *et al.*, 1964; Hungate, 1966; Stewart and Bryant, 1988).

The dual-flow continuous culture model system does have limitations, including loss of protozoal populations, shift in archaeal population from *Methanobacteriaceae* to *Methanomicrobiales* (Sharp *et al.*, 1998) and changes in subgroup structure of *Fibrobacter* populations. However, to date, no other rumen model operates with turnover rates as close to physiological or, with high feed intakes, modelling not only ruminal digestion, but also fermentation

characteristics. In addition, neither protozoal nor bacterial populations are maintained at *in vivo* levels in these models (Mansfield *et al.*, 1995; Prevot *et al.*, 1995). The concentration of rRNA (μg of SSU rRNA g^{-1} of sample) in the fermenter samples was similar to that in ruminal samples and indicated that the fermenters were able to maintain a microbial biomass similar to the naturally occurring microbial biomass in the rumen. The model rumen was able to maintain important microbial groups, both at lower (*Fibrobacter*) and upper (Archaea) levels of the ruminal community trophic structure. The ability of the fermenters to maintain these microbial populations in proportions and amounts of SSU rRNA similar to those found in the rumen, in combination with the similarities in fermentation characteristics and substrate digestion, indicate that the model system maintains a functional community structure similar to the rumen. By maintaining a stable simulation of rumen function and gross microbial population structure, the dual-flow continuous culture fermenter system could provide a tool for elucidating some relationships and interactions that occur among ruminal microorganisms.

Experimental procedures

The model rumen system was evaluated in two experiments by two comparative criteria. The initial experiment measured temporal changes in the population structure during the adaptation of ruminal microbial populations in the fermenters (adaptation of microbial populations to model rumen). Divergence between ruminal and fermenter population structure was evaluated in the second experiment (comparison of model and natural rumens). Experimental diets, inoculum harvest and sample processing were the same for both experiments; sampling times and populations targeted by oligonucleotide probes were different in each experiment. All chemicals used in both experiments were purchased from Sigma (Sigma Chemical) unless otherwise noted.

Diet and inoculum harvest

The experimental diet was 60% chopped alfalfa hay and 40% concentrate (36.8% ground corn, 1.5% soybean meal and 1.7% salt, minerals and vitamins) and was balanced to meet or exceed National Research Council recommendations for a lactating cow producing 36.5 kg of milk d^{-1} (National Research Council, 1989). The diet fed to fermenters was ground to pass through a 2 mm screen and pelleted (6 mm diameter $\times$ 10 mm length) in a laboratory pellet mill (California Pellet Mill) to facilitate automatic feeding.

A lactating, multiparous Holstein cow, with ruminal cannula fitted according to guidelines stipulated by the University of Minnesota Animal Care and Usage Committee, served as the inoculum donor. This animal was fed the experimental diet, twice daily, for 14 d prior to inoculum harvest. Inoculum was harvested from the rumen 3–4 h after the morning feeding using a vacuum pump with attached hose adapted to inhibit collection of large particles [24 holes (0.5 cm diameter) drilled in the lower 15 cm and the end plugged]. The hose was moved around the reticulorumen to facilitate representative sampling. Approximately 5 l of ruminal contents (primarily fluid) was collected, strained through two layers of cheesecloth and placed in a prewarmed insulated container (approximately 11 l volume) for transport to the laboratory.

Fermenter inoculation and operation

The operation of the dual-flow continuous culture fermenters has been described by Hannah *et al.* (1986) and is described here in brief. The temperature of the fermenters was maintained at 38°C, the liquid dilution rate was 0.10 h^{-1}, the solid dilution rate was 0.055 h^{-1}, pH was controlled at 6.25 $\pm$ 0.05 and continual nitrogen gas infusion was used to maintain anaerobic conditions. Mineral buffer solution (Weller and Pilgrim, 1974) containing 0.5 g urea l^{-1} was infused continuously into the fermenter flasks. Fermenter contents were mixed continuously and daily dry matter addition was 75 g, fed automatically in eight equal feedings d^{-1}. Fermenter effluent containers were kept in a 4°C waterbath during the sampling period in order to inhibit biological activity. To ensure homogeneous inoculation of the fermenters, the insulated container was manually agitated to mix the contents immediately before inoculation and ruminal contents were added to four fermenters in approximately 250 ml aliquots until fluid reached the overflow tube (about 1000 ml). After inoculation, fermenter contents were mixed and immediately sampled for nucleic acid extraction using a sterile 10 ml wide-bore pipette. Dietary dry matter (25 g) was added to fermenters after inoculation and the automatic feeding system was then started.

Sampling

Adaptation of microbial populations to the model rumen. Samples for nucleic acid extraction were obtained from the rumen and from fermenters after 0, 6, 12, 24, 48, 72, 96, 120, 168 and 240 h of operation. A second sample was obtained from one fermenter at each time-point and the duplication was rotated through all fermenters (i.e. at 0 h, a duplicate sample was taken from Fermenter 1; at 6 h, a duplicate sample was taken from Fermenter 2, etc.) in order to facilitate statistical analysis.

Comparison of model and natural rumens. Ruminal samples for nucleic acid extraction and chemical fermentation characteristics were taken at the time of inoculum collection and then on three further consecutive days. The cow (inoculum donor) was sampled at the same time each day, 3–4 h after feeding. Samples from the rumen were taken from a composite of ruminal contents from five sites in the rumen (central, cranial dorsal, cranial ventral, caudal dorsal and caudal ventral). Samples were taken from the fermenters at 0, 96, 120, 144 and 168 h.

Sample processing. Samples for rRNA extraction were taken as follows. An aliquot (0.3 ml) of fermenter contents was placed into a preweighed 2.2 ml screw-cap tube (Sarstead) containing 0.3 g of zirconium beads (0.1 mm

diameter) that had been baked at 300°C overnight to inactivate RNases. Immediately after adding the sample, tubes were frozen in dry ice and stored at −80°C.

Fermenter effluent was collected at each sampling time-period (except for 0 h) for VFA and ammonia-nitrogen (NH₃-N) determinations. Ammonia nitrogen was determined by steam distillation with a Kjeltec Autoanalizer (Tecator) using magnesium oxide, instead of sodium hydroxide, as the base (AOAC, 1984). Volatile fatty acid samples were prepared by the method of Erwin *et al.* (1961) and analysed by gas chromatography (Hewlett Packard Model 5880 A with a 80/120 carbopack DA/4% carbowax 20 M column, Hewlett Packard). Nutrient digestibilities were determined as previously described (Mansfield *et al.*, 1995) using approved methodologies (AOAC, 1984).

RRNA extraction and oligonucleotide probe hybridization

DNA oligonucleotide probes, complementary to small subunit (SSU) rRNA, were used to determine the general microbial community structure of the samples (Raskin *et al.*, 1994b). Synthesis and labelling (with ^{32}P) of the oligonucleotide probes followed the procedures of Zheng *et al.* (1996). Probes, target groups and reference organisms are shown in Table 4. The first five probes were used to evaluate the adaptation of microbial populations to the model rumen and all 10 probes were used to compare model and natural rumens. Extraction of RNA used phenol and mechanical disruption (i.e. bead beating), as described by Sharp *et al.* (1998). The quality of extracted rRNA was evaluated using polyacrylamide gel electrophoresis (Mighty Small II Slab Gel Electrophoresis Unit, Hoefer Scientific Instruments) and the concentration of nucleic acid was determined spectrophotometrically. Nucleic acids were denatured and diluted to 1.5 ng μl^{-1} as previously described (Raskin *et al.*, 1994b). Samples were applied in triplicate, 50 μl slot^{-1}, to Magna Charge membranes (Micron Separation) using a slot blot device (Minifold II, Schleicher and Schuell) under a slight vacuum to pull the entire sample through the membrane in 1–2 min. Membranes were air-dried and then baked for 2 h at 80°C.

Baked membranes were pre-wetted in hybridization buffer [0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, 10× Denhardt solution (Sambrok *et al.*, 1989), 0.5% SDS, 0.05 mg poly (A) ml^{-1}] and placed in hybridization tubes (4 cm diameter × 28 cm length for universal probe and 4 cm diameter × 14 cm length for all other probes; Robbins Scientific). Membranes were incubated with approximately 10 ml of hybridization buffer for 2 h at 40°C in a rotating incubator (Robbins Scientific). All hybridization incubations for the Gram-positive bacteria probe were carried out at room temperature. The first hybridization buffer was discarded and the labelled probe was added by inclusion in a volume of hybridization buffer equal to that used for initial incubation. Incubation was then continued at 40°C for 16–20 h. Membranes were then washed in the hybridization tubes with 100 ml of 1% SDS/1× SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 1.0) for 2 h at 40°C, replacing the wash solution after 1 h. Membranes were then removed from the hybridization tubes and washed twice for 15 min in 300 ml of 1% SDS/1× SSC at the experimentally determined dissociation temperatures for individual probes (Stahl and Amann, 1991).

Air-dried membranes with bound probe were exposed to Storage Phosphor screens (Molecular Dynamics) to quantify the retained [^{32}P]-labelled probe using a 400-A Phosphor-Imager (Molecular Dynamics) to scan the exposed screens, and image analysis was carried out with the IMAGEQUANT software package (Molecular Dynamics). Standard curves were calculated from reference RNA by linear regression (Neter *et al.*, 1985). Abundances of specific groups of organisms are presented as percentages of total SSU rRNA in the sample, as previously described by Raskin *et al.* (1994a).

Statistical analysis

Adaptation of microbial populations to model rumen. Duplicate samples from each time-period were used to determine standard deviations for hybridization data. For each probe, results from the fermenters at each time-point were compared with those in ruminal samples using the Student's *t*-test (Neter *et al.*, 1985). Significance for both tests was $P < 0.05$. Nutrient digestion and fermentation characteristics data were used as a check of fermenter operation and therefore not analysed statistically and are presented as means of the four fermenters.

Table 4. List of DNA oligonucleotide probes, target organisms and reference organism used.

Probe[a]	Target	Reference organism	Reference
S-*-University of-1392-a-A-15	All known organisms	*Escherichia coli*	Stahl and Amann, 1991; Weller and Pilgrim, 1974
S-D-Bact-0338-a-A-18	Bacteria	*Bacteroides thetaiotaomicron*	Amann *et al.*, (1990)
S-D-Euc-0516-a-A-16	Eukarya	*Saccromyces cervisae*	Hicks *et al.*, (1992)
S-D-Arch-0915-a-A-21	Archaea	*Methanosarcia acetivorans*	Raskin *et al.*, (1994a)
S-G-Fibr-0225-a-A-21	*Fibrobacter* genus	*Fibrobacter succinogenes* AC3	Stahl and Amann, 1991
S-P-Gpos-1200-a-A-13	Gram-positive bacteria	*Streptococcus bovis*	Lin and Stahl, 1995
S-S-F.suc-0650-a-A-20	*F. succinogenes*	*F. succinogenes* AC3	Amann *et al.*, 1992
S-S-F.int-0136-a-A-20	*F. intestinalis*	*F. intestinalis* DR7	Lin *et al.*, 1994
S-Ss-F.s.suc-0628-a-A-22	*F. succinogenes* subgroup 1	*F. succinogenes* BL2	Lin *et al.*, 1994
S-Ss-F.s.suc(3)-0628-a-A-22	*F. succinogenes* subgroup 3	*F. succinogenes* HM2	Lin *et al.*, 1994

a. First five probes listed were used for adaptation to model rumen study and all 10 probes were used for model and natural rumen comparison.

Comparison of model and natural rumens. For purposes of analysis, the four samples obtained from the animal were designated as ruminal samples, the four 0 h fermenter samples were designated as inoculum samples and the other samples taken from fermenters were designated, by time of fermenter operation, as 96, 120, 144 and 168 h samples, four samples for each time-point. Chemical fermentation characteristics data were determined for ruminal and fermenter samples and analysed by analysis of variance using the General Linear Models procedure of SAS Institute Inc. (1988). Data are presented as least square means, and error bars on figures represent standard error of the means (SEM). Paired *t*-tests were used to compare ruminal, inoculum, 96, 120, 144 and 168 h samples. Differences among fermenters were also evaluated using paired *t*-tests. The paired *t*-test procedure of SAS Institute Inc. (1988), Proc Ttest, was used and the level of probability of the *t* statistic was calculated with the Cochran and Cox approximation (SAS Institute Inc., 1988). The level of probability set for significance was $P < 0.05$. Data are presented as the means of four samples, and error bars represent the SEM.

Acknowledgements

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